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Dietary and supplemental maternal methyl-group donor intake and cord blood DNA methylation

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Abstract

Maternal nutrition is critically involved in the development and health of the fetus. We evaluated maternal methyl-group donor intake through diet (methionine, betaine, choline, folate) and supplementation (folic acid) before and during pregnancy in relation to global DNA (hydroxy)methylation and gene specific (*IGF2 DMR, DNMT1, LEP, RXRA*) cord blood methylation. 115 mother-infant pairs were enrolled in the MAternal Nutrition and Offspring's Epigenome (MANOE) study. The intake of methyl-group donors was assessed using a food-frequency questionnaire. LC-MS/MS and pyrosequencing were used to measure global and gene specific methylation, respectively. Dietary intake of methyl-groups before and during pregnancy was associated with changes in *LEP*, *DNMT1*, and *RXRA* cord blood methylation. Statistically significant higher cord blood *LEP* methylation was observed when mothers started folic acid supplementation more than 6 months before conception compared to 3-6 months before conception (34.6±6.3%vs.30.1±3.6%,p=0.011, *LEP* CpG1) or no folic acid used before conception (16.2±4.4%vs.13.9±3%,p=0.036 for *LEP* CpG3 and 24.5±3.5%vs.22.2±3.5%,p=0.045 for *LEP* mean CpG). Taking folic acid supplements during the entire pregnancy resulted in statistically significantly higher cord blood *RXRA* methylation as compared to stopping supplementation in the second trimester (12.3±1.9%vs.11.1±2%,p=0.008 for *RXRA* mean CpG). To conclude, long-term folic acid use before and during pregnancy was associated with higher *LEP* and *RXRA* cord blood methylation, respectively. To date, pregnant women are advised a folic acid supplement of 400 µg/day from 4 weeks before until 12 weeks of pregnancy. Our results suggest significant epigenetic modifications when taking a folic acid supplement beyond the current advice.

Key words: methyl donors, folic acid supplementation, preconception, pregnancy, DNA methylation, *LEP, RXRA, DNMT1, IGF2*

Introduction

Maternal nutrition is critically involved in the growth, development, and health of the fetus. This has been most clearly shown in studies from the Dutch Hunger Winter (1944 - 1945). Severe cold and wartime resulted in a 5-month period of extreme food shortage in the Netherlands. Long-term follow-up studies from this cohort found that adults who had been exposed to the famine early in gestation showed low birth weight and increased risk of obesity as adults.[1, 2] This adaptive process in response to famine (nutritional insult) during a vulnerable period early in life is known as fetal metabolic programming.[3] One of the underlying mechanisms responsible for fetal programming are epigenetic modifications, such as DNA methylation.[4] DNA methylation takes place when a methyl-group (CH_3) is added to the carbon-5 position of cytosine in CpG dinucleotides.[5] Methylation of gene promoters and regulatory regions hinder the binding of transcription factors, leading to altered gene expression.[6] The One-Carbon (I-C) metabolism plays a central role in DNA methylation since it determines the flux of methyl-groups towards methylation of DNA. Folate, betaine, choline, and methionine are the main sources of dietary methyl-group donors in the I-C metabolism. Folic acid is the synthetic form of folate used in supplements and for food fortification. Women who wish to become pregnant are advised to take a folic acid supplement of 400 µg per day starting 4 weeks before conception until 12 weeks of pregnancy for the prevention of neural tube defects.[7] All methyl-group donors enter the I-C metabolism at different sites and are, in the end, all converted to the universal methyl-group donor S-adenosylmethionine (SAM).[8] The transfer of methyl-groups from SAM to the DNA is catalyzed by three DNA methyltransferases (*DNMT*s), with *DNMT1* as a maintenance *DNMT* which is required to maintain DNA methylation patterns.[5] *DNMT3A and 3B* are responsible for the establishment of new or de novo DNA methylation patterns starting from fertilization until implantation. During this short period, DNA methylation marks on the maternal and paternal genome are globally de- en remethylated. Later in pregnancy, during organogenesis and tissue differentiation, there is a progressive increase in DNA methylation. Thus, there are several critical windows during fetal development where dietary factors can influence the fetal epigenome.[9]

Therefore, to assess the effect of maternal nutrition during pregnancy on offspring DNA methylation levels, maternal dietary information and supplement use should be obtained at several time points during pregnancy (early, mid, and late gestation), because offspring DNA methylation levels and health risk are found to be different depending on the time of exposure during gestation. For example, individuals who were exposed to famine early in gestation (Dutch Hunger Winter) showed 5.2 % lower methylation levels at the insulin-like growth factor 2 (*IGF2*) differentially methylated region (DMR) as compared to non-exposed siblings. Exposure in late gestation on the other hand, showed decreased glucose tolerance and no difference in *IGF2 DMR* methylation between exposed and non-exposed siblings.[2, 4] *IGF2* is a maternally imprinted gene that is regulated by two differentially methylated regions (DMRs) and is important for fetal growth and development. Since the *IGF2 DMRs* are only methylated on the maternal allele, this region might be particularly susceptible to nutritional insults and supplementation in the pre- and periconceptional period.[10] Another study from the Dutch Hunger Winter found a significant increase in leptin (*LEP*) methylation of adults (men only) exposed to famine in early and late gestation.[11] *LEP* produces the hormone leptin, which is involved in food intake (inhibition) and energy expenditure, and thus a regulator of body weight. *LEP* promoter DNA methylation has been linked to adverse pregnancy outcomes and is plausibly involved in fetal metabolic programming.[12] Another metabolic gene that can be affected by maternal nutrition is the retinoid X receptor alpha (*RXRA)* gene*,* which is known to be involved in insulin sensitivity, adipogenesis, and fat metabolism. Lower maternal carbohydrate intake in early pregnancy was associated with higher *RXRA* cord blood methylation and with greater offspring's adiposity (fat mass and percentage fat mass) in 9-year old children.[13]

Besides changes in maternal diet, several studies have shown that maternal supplement use can induce alterations in offspring epigenetic marks. For example, periconceptional folic acid use of 400 µg/day was associated with a 4.5 % increase in *IGF2 DMR* methylation in infants (17 months old), compared to children who were not exposed to folic acid. [14] However, according to Hoyo et al.[15] no differences in cord blood *IGF2* methylation of infants born to women taking no, moderate (400 –

1000 µ/day), and high doses (> 1000 µg/day) of folic acid before and during pregnancy were found. Maternal choline supplementation during pregnancy has also been shown to modify the neonate epigenome. One study examined the effect of choline intake (480 mg vs. 930 mg/day) in the third trimester of pregnancy on offspring DNA methylation. They found a decrease in placental DNA methylation of cortisol regulating genes (*CRH* and *NR3C1*) with higher maternal choline intake. Global DNA methylation and site-specific DNA methylation (*LEP*, *IL10*, *IGF2*, and *GNASAS1*) was however not altered by maternal supplemental choline intake.[16] The long-term effects of these methylation changes, due to maternal supplementation, on offspring health remain unknown.

In this study, we aimed to determine the effect of maternal dietary methyl-group donor intake (methionine, folate, choline, and betaine) and supplemental intake (folic acid) before and during each trimester of pregnancy on global DNA (hydroxy)methylation and gene specific methylation in cord blood. Promoter regions of *RXRA, LEP*, and *DNMT1,* and *IGF2 DMR* were selected for gene specific DNA methylation analysis.

Results

Maternal and neonatal characteristics

From the 115 mothers, mean maternal age was 31 years (range: 25 - 41), mean BMI was 23.1 ± 3.4 kg/m^2, and mean gestational weight gain was 14.8 kg (range: 1.9 - 28.9) (table 1). Only five women smoked before and during the first trimester of pregnancy. Three of them continued smoking during the second and third trimester. The newborns, of which 55 were girls (47.8 %), had a mean birth weight of 3518 ± 405.4 g and mean gestational age of 39.6 ± 0.9 weeks. Birth weight-for-gestational age z-scores were calculated and a mean z-score of 0.57 ± 0.93 was obtained (range: -1.38 - 2.91).

The mean maternal intake of dietary methyl-group donors before and during each trimester of pregnancy is presented in table 2. The intake of dietary methyl-group donors was stable during the course of pregnancy. Supplemental intake of folic acid before and during each trimester of pregnancy

is presented in table 3. Folic acid intake was significantly higher in the first trimester of pregnancy (504.6 µg, p = 0.000), compared to the intake before (371.5 µg) or during the other trimesters of pregnancy (386.9 and 3564 µg). Women are advised to take a folic acid supplement four weeks prior to conception. The majority of women in our study followed this guideline, however 25.2 % (n = 29) did not take a folic acid supplement before conception. 26.1 % (n = 30) on the other hand, took a folic acid supplement more than 6 months prior to conception. Most women (43.8 %) stopped the folic acid supplementation in the second trimester, but 38.3 % of the women took the supplement during their entire pregnancy.

Cord blood DNA methylation levels

The 115 newborns had a mean global DNA methylation level of 6.51 ± 1.65 % and a mean global DNA hydroxymethylation level of 0.23 ± 0.14 %. The mean methylation percentage of *IGF2* DMR, *DNMT1*, *LEP*, and *RXRA* was 51.39 ± 4 %, 1.53 ± 0.3 %, 22.91 ± 3.36 %, and 11.73 ± 1.97 % respectively.

Impact of dietary methyl-group donor intake before and during each trimester of pregnancy on cord blood DNA methylation

We next determined the effect of maternal dietary methyl-group donor intake before and during each trimester of pregnancy on offspring global DNA (hydroxy)methylation and gene specific methylation. Associations between maternal dietary methyl-group donor intake and cord blood methylation are presented in table 4. Before pregnancy, higher intakes of betaine and methionine were associated with higher cord blood methylation levels of *DNMT1* CpG4 (0.68 % per 100 mg increase, 95 % CI: 0.04 - 01.31, p = 0.039) and *LEP* CpG4 (0.43 % per 100 mg increase, 95% CI: 0.01 - 0.85, p = 0.048), respectively. In the second trimester of pregnancy, high methyl-group donor intakes (except for methionine) were negatively associated with gene specific cord blood methylation

(betaine with *LEP* CpG2; choline with *DNMT1* CpG4; folate with *LEP* CpG2 and *DNMT1* CpG4). In the last trimester of pregnancy, a high intake of choline and folate was associated with higher methylation levels of *DNMT1* CpG2 (0.29 % per 100 mg increase, 95 % CI: 0.1 - 0.84, p = 0.022) and lower methylation levels of *RXRA* CpG2 (-1.001 % per 100 µg increase, 95 % CI: -1.96 - -0.04, p = 0.041), respectively. Finally, no significant associations between maternal dietary methyl-group donor intake before and during pregnancy and cord blood global DNA (hydroxy)methylation, *IGF2* DMR methylation (CpG1, CpG2, CpG3, and mean CpG), and birth weight were found (data not shown). In addition, no associations between maternal dietary methyl-group intake in the first trimester and cord blood methylation were found.

Impact of folic acid intake before conception on cord blood DNA methylation

We found statistically significant differences in cord blood *LEP* methylation (CpG1, CpG3, and mean CpG) by duration of maternal folic acid intake before conception (no folic acid use before conception/ 1 - 3 months prior to conception/ 3 - 6 months prior to conception /> 6 months prior to conception). The results are shown in figure 1. For *LEP* CpG1, we found a statistically significant difference between the four groups (p = 0.029). A post hoc test revealed that the methylation percentage was significantly higher when the mother used a folic acid supplement more than 6 months before conception (34.6 ± 6.3 %, p = 0.011) compared to 3 to 6 months before conception (30.1 ± 3.6 %). Also *LEP* CpG3 (p = 0.037) and mean *LEP* CpG (p = 0.024) methylation percentages showed significant differences: significantly higher methylation levels were seen when women took a folic acid supplement more than 6 months prior to conception compared to no folic acid use, i.e. 16.2 ± 4.4 % vs. 13.9 ± 3 % (p = 0.036) for LEP CpG3 and 24.5 ± 3.5 vs. 22.2 ± 3.5 % (p = 0.045) for mean *LEP* CpG.

Impact of folic acid intake during pregnancy on cord blood DNA methylation

We found statistically significant differences in cord blood *RXRA* methylation (CpG1, CpG2, CpG3, CpG4, CpG5 and mean CpG) by duration of maternal supplemental folic acid intake during pregnancy (stop folic acid supplement intake at the end of the first trimester, stop in the second trimester, stop at the end of the third trimester). The results are shown in figure 2. For all five CpG's and the mean CpG, we found statistically significant differences between the 3 groups (CpG1, p = 0.027; CpG2, p = 0.012; CpG3, p = 0.009; CpG4, p = 0.024; CpG5, p = 0.037; mean CpG, p = 0.01). Post hoc tests revealed that *RXRA* methylation percentages - in all CpG's (except CpG5) and mean CpG - were significantly higher in mothers using a folic acid supplement during the whole pregnancy compared to stopping the supplementation after the first or second trimester. The mean %, standard error of the mean, and p-value for CpG1, CpG2, CpG3, CpG4, and mean CpG were 8.1 ± 1.3 % vs. 7.3 ± 1.6 % (p = 0.02); 27.1 ± 4.1 % vs. 24.4 ± 4.7 % (p = 0.009); 8.2 ± 1.2 % vs. 7.4 ± 1.5 % (p = 0.008); 8.6 ± 1.8 % vs. 7.7 ± 1.9 % (p = 0.05); 12.3 ± 1.9 % vs. 11.1 ± 2 % (p = 0.008) respectively.

Discussion

This study supports the hypothesis that maternal methyl-group donor intake before and during pregnancy can induce epigenetic modifications in offspring genes related to metabolism.

We first studied the effect of **supplemental folic acid intake before conception** on cord blood methylation (global DNA (hydroxy)methylation, *IGF2* DMR, *LEP, RXRA,* and *DNMT1)*. It is recommended that women, who desire to become pregnant, use a folic acid supplement of 400 µg/day starting 4 weeks before conception until 12 weeks of pregnancy for the prevention of spina bifida.[7] In reality, women often start the folic acid supplementation months before conception, exposing the fetus to high levels of circulating folic acid during early embryonic development. We found statistically significant differences in cord blood *LEP* methylation depending on the start of the folic acid supplementation before conception. Specifically, a higher *LEP* methylation was observed

when folic acid supplementation started more than 6 months prior to conception (24.5 ± 3.5 %) compared to no preconceptional folic acid use (22.2 ± 3.5 %). *LEP* is primarily expressed in white adipose tissue and its product, the hormone leptin, has several functions including regulation of food intake (inhibition), body weight, energy homeostasis, and it is expressed and secreted by the placenta during pregnancy.[17] It has been shown that the *LEP* promoter is subject to epigenetic programming and that the expression of leptin can be modulated by DNA methylation.[18] For example, in utero exposure to famine and gestational diabetes have been associated with offspring *LEP* promoter hypermethylation in blood of adults[11] and placental *LEP* hypermethylation[19], respectively. According to Lesseur et al.[12], cord blood *LEP* methylation was higher in small for gestational age infants and lower in infants born to pre-pregnancy obese mothers. Modifications in the profile of leptin in early life may contribute to the lower expression of appetite regulators, alter fetal neural development, and in the end alter the susceptibility to obesity and metabolic disorders in adulthood.[18]

We also studied the effect of **dietary methyl-group donor intake before conception** on cord blood DNA methylation. Higher intake of methionine and betaine before conception were associated with higher methylation levels at *LEP* CpG4 and at *DNMT1* CpG4, respectively. *DNMT1* produces the enzyme DNA methyltransferase, which maintains DNA methylation in newly synthesized DNA strands.[5] Animal studies[20-22] have shown that maternal diet can influence *DNMT1* methylation/expression. For example, a choline deficiency in pregnant rats hypomethylates the regulatory CpG's within the *DNMT1* gene, leading to its overexpression and this results in an increase of global and gene specific (*IGF2*) DNA methylation.[20]

The periconceptional period may be particularly susceptible to methyl-group donor intake due to global de- and remethylation of the embryonal DNA in early development (between fertilization and implantation). However, our and other results show that there are different windows of susceptibility (organogenesis and tissue differentiation) to epigenetic modifications by gestational methyl-group donor intake and that the focus should not be solely on the periconceptional period.[23]

Next, we studied the effect of **supplemental folic acid intake during each trimester of pregnancy** on cord blood DNA methylation. We found statistically significant difference in cord blood *RXRA* methylation depending on the duration of folic acid intake during pregnancy. *RXRA* methylation was significantly higher (12.3 ± 1.9 %) when the mother used a folic acid supplement during the whole pregnancy compared to stopping the supplementation in the second trimester (11.1 ± 2 %). The *RXRA* gene is known to be involved in insulin sensitivity, adipogenesis, and fat metabolism. In two independent cohorts, Godfrey and colleagues found that higher *RXRA* methylation in umbilical cord tissue at birth, was highly correlated with adiposity (fat mass and percentage fat mass) in 9-year-old children. In one of these cohorts, low maternal carbohydrate intake in early pregnancy was associated with higher *RXRA* methylation.[13] This study showed that *RXRA* DNA methylation levels at birth could provide information about prenatal environmental influences, and later phenotype (adiposity). In the current MANOE cohort, folic acid supplementation during the entire pregnancy resulted in higher cord blood *RXRA* methylation. Children from the MANOE cohort will be further followed-up in the context of high vitamin intake by mothers, epigenetic modifications in cord blood, and obesity/metabolic disorders in childhood (BMI, fat content). Although it is widely known that folate intake reduces the risk of neural tube defects[7], the potential long-term consequences of an increased folate intake are largely unknown in humans. One study in humans found no effect of supplement use up to 12 weeks of pregnancy (current recommendations) on cord blood methylation. However, supplement use after 12 weeks of gestation was previously associated with higher methylation in the gene *IGF2*, and lower *PEG3* gene and LINE-1 total DNA methylation in cord blood.[24] Our data suggest significant epigenetic modifications in the examined metabolic genes when taking a folic acid supplement beyond the current advice.

Finally, we studied the effect of **dietary methyl-group donor intake during each trimester of pregnancy** on cord blood DNA methylation. The intake of dietary methyl-group donors during pregnancy was found to be associated with *LEP, RXRA*, and *DNMT1* cord blood methylation, but not with global DNA (hydroxy)methylation and *IGF2 DMR* methylation. Only negative associations

between dietary methyl-group donor intake and cord blood methylation were found in the second trimester of pregnancy, positive associations were observed for the other time points. A possible explanation for this shift could be a change in the I-C metabolism during gestation. A higher rate of transsulfuration was previously reported in the first trimester of pregnancy and a higher rate of transmethylation in the third trimester.[25] At each time point, we found that the intake of methyl-group donors was associated with *DNMT1* methylation. One possible mechanism that leads to changes in *LEP* and *RXRA* methylation could be via alterations in the methylation and thus gene expression of *DNMT1*.[20] In this study positive, negative, and no associations between maternal methyl-group donor intake and offspring DNA methylation levels were found. It seems that there is no simplistic correlation between maternal methyl-group donor intake and offspring DNA methylation. Other studies also did not find a linear relationship, for example under nutrition (which correlates with reduced methyl-group donor availability) resulted in a decrease and increase of different site-specific genes.[4, 11, 26-28]

There are some strengths and limitations in the present study we need to address. The strengths of the present study include a unique study design that allowed us to collect longitudinal maternal data (starting before pregnancy and during each trimester of pregnancy), and offspring global and gene specific DNA methylation data in cord blood. The use of a validated food-frequency questionnaire designed to assess the intake of the nutrients under study. In addition, at each study time point detailed information about supplement use was obtained. We have detailed covariate data allowing for adjustment for potential confounding variables. Another advantage is the use bisulfite pyrosequencing for DNA methylation analysis in candidate genes. It enables the determination of DNA methylation levels at individual CpG sites and the calculation of the average methylation percentage of that region. Single CpG site methylation in the promoter region of a gene can be involved in the regulation of transcription, especially when it lies in a relevant transcription factor binding site, and could be associated with diseases. From example, the loss of DNA methylation in one CpG site in the promoter region of *TET1* was associated with air pollution and childhood asthma;

and could possibly be a potential biomarker for childhood asthma.[29] CpG methylation within the same CpG island in promoter regions have shown to be highly correlated and these methylation patterns have been shown to differ from methylation patterns elsewhere, indicating that they have a specific biological role.[30]

A first limitation is that we measured offspring methylation using cord blood, which is composed of different cell types, each with a different DNA methylation profile. Cord blood might not be the target tissue of interest for long-term metabolic outcomes, but is most often used in epidemiological studies because it is easy to obtain. In addition, cord blood consists primarily of infant blood[31] and can be considered as a good surrogate for the newborns blood epigenome. Another limitation is the fact that the Belgian food composition database NUBEL[32] does not contain information about the four methyl-group donors under study. Databases of neighboring countries or the USDA database for choline and betaine[33] content were used in the validation of the FFQ.[34, 35] For folate, the Dutch NEVO food composition database was used[36] and the German BLS Nutrient database for methionine.[37] The USDA database was also used for the nutrient content of folate and methionine if not found in NEVO and BSL databases respectively.

To conclude, this study shows that maternal methyl-group donor intake (through diet and supplement use) before and during each trimester of pregnancy can influence offspring DNA methylation in genes related to metabolism. Especially, long-term folic acid use before or during pregnancy was associated with higher *LEP* and *RXRA* cord blood methylation levels. Our results suggest significant epigenetic alterations in the genes under study when not following the current advice for pregnant women on folic acid supplementation between 4 weeks before until 12 weeks pregnancy. However, the impact these methylation changes may have on (later) health are yet to be determined.

Methods

Study subjects

We studied participants enrolled in the MANOE (MAternal Nutrition and Offspring's Epigenome) study, an ongoing prospective, observational cohort study initiated in April 2012. We enrolled 150 women (34 women before pregnancy and 116 in the first trimester of pregnancy) between April 2012 and January 2015 at the Department of Obstetrics and Gynecology of the University Hospitals Leuven (Belgium). The last delivery of the cohort took place in September 2015. Of the 150 enrolled women, 35 mother-infant pairs were excluded from analysis due to either missing nutritional data (n = 2), a missing cord blood sample (n = 14), development of pregnancy complications (gestational diabetes (n = 8) and preeclampsia (n = 1)), pre-term delivery (n = 6), extreme high intake of folic acid ($\geq$ 4 mg/day) (n = 2), or birth defects (n = 2). After these exclusions, 115 mother-infant pairs were available for statistical analysis. The recruitment process has been described in more detail in a previous study [38].

Maternal and Neonatal Measurements

All 115 women were followed-up during pregnancy at their scheduled ultrasounds (11 - 13 weeks, 18 - 22 weeks, and 30 - 34 weeks of gestation) and at delivery. From the women recruited before pregnancy (n = 27) we obtained extra measurements before conception. A food-frequency questionnaire (FFQ) was developed and validated to assess maternal intake of dietary methyl-group donors (methionine, folate, betaine, and choline) before and during each trimester of pregnancy.[34, 35] 24 FFQ's were obtained before pregnancy, 96 FFQ's at 11 - 13 weeks, 89 FFQ's at 18 - 22 weeks, and 83 at 30 - 34 weeks of pregnancy. To assess the intake of methyl-group donors through supplement use, questions were asked about the use of nutritional supplements (frequency, brand/type, dosage) before and during each trimester of pregnancy. Only the intake of folic acid (synthetic form of folate) was registered, since there was no report on the supplemental intake of methionine, betaine, and choline. Furthermore, using a combination of questionnaires and interviews, we collected

information about a range of socio-demographic factors, life style habits, and physical activity. Information on mothers' smoking status before and during pregnancy was obtained at each consultation. Questions were asked about smoking before and in each trimester of pregnancy and the number of cigarettes smoked on average per day. From these data, a dichotomous variable for maternal smoking before and during pregnancy was derived (smoked/did not smoke). Height and pre-pregnancy weight was used to calculate the Body Mass Index (BMI, kg/m²). Maternal measurements have been described in more detail in a previous paper.[38]

Determination of gestational age was based on the crown rump length measured between 7 and 14 weeks of gestation in all patients.[39] We obtained birth weight and length from the hospital clinical record. Gender specific z-scores for birth weight for gestational age were generated using the INTERGROWTH-21st tool.[40]

Sample collection and DNA extraction

At delivery, we collected umbilical cord blood in 4.5 mL tubes containing EDTA (BD Vacutainer Systems) via venipuncture. Umbilical cord blood samples were put in the freezer (-20°C) immediately after collection. DNA from whole blood was extracted with the Salting out method[41], the quantity and purity of DNA were determined by a NanoDrop spectrophotometer. Samples were stored at -80°C until analysis.

Global DNA (hydroxy)methylation measurements

Cord blood DNA of 115 infants were analyzed by fast and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantification of 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) as described previously.[42] Briefly, isolated genomic DNA samples (10 µg) were hydrolyzed to individual deoxyribonucleosides by a simple one-step DNA hydrolysis procedure. For this, a digest mix was prepared by adding phosphodiesterase I, alkaline phosphatase and Benzonase Nuclease to Tris-HCl buffer. Extracted DNA was then hydrolyzed by

adding 10 µL digest mix and incubating at 37°C for at least 8 h. After hydrolysis, 490 µL of acetonitrile/water was added to each sample. Global DNA methylation and hydroxymethylation was obtained by quantifying 5-mC, 5-hmC and C using ultra-pressure liquid chromatography (UPLC), in combination with tandem mass spectrometry (MS-MS). Global DNA methylation was expressed as a percentage of 5-mC over the sum of 5-mC, 5-hmC and C (% Global DNA Methylation = ((5-mC / 5-mC + 5-hmC+ C)*100). Global DNA hydroxymethylation was expressed as a percentage of 5-hmC over the sum of 5-mC, 5-hmC and C (% Global DNA Hydroxymethylation = ((5-hmC / (5-mC + 5-hmC + C)*100).

Gene specific DNA methylation measurements

Gene and region selection

We adopted a candidate gene approach and consulted previously published EWAS data, thus selecting candidate genes based on a literature study. We selected 12 genes that are known to be involved in the onset of obesity, genes of which the DNA methylation state is nutrient sensitive, or genes involved in DNA (de)methylation reactions. In a first phase, we analyzed offspring DNA methylation of the 12 selected genes on a subsample (n = 30). The subsample was selected based on maternal methyl-group donor intake (low versus high intake). After statistical analysis, we selected 4 genes (*DNMT1*, maintenance of DNA methylation patters; *LEP,* appetite control*; RXRA*, role in insulin sensitivity, adipogenesis, and fat metabolism; and *IGF2* DMR, growth) to test our hypothesis on the entire cohort. For *IGF2* DMR, DNA methylation was measured at CpGs in the DMR that regulates parental imprinting of the IGF2 gene in early development. For the other three genes, we have selected CpGs within the promoter region, since epigenetic changes in these regulatory regions can influence gene expression.

Bisulfite Conversion and PCR

Genomic DNA (200 ng) was bisulfite converted using the EZ-96 DNA Methylation-Gold™ Kit (#D5008, Zymo Research). Converted DNA was eluted with 30 µL of M-elution buffer. Subsequently, 1 µL of

converted DNA was amplified by PCR in a total volume of 25 µL containing 0.2 µM of primers and 2x Qiagen PyroMark PCR Master Mix (#978703, Qiagen). Primers for *DNMT1, RXRA,* and *LEP* were ordered from Qiagen (#PM00075761, #PM00144431, #PM00129724 PyroMark CpG Assays).

Primer sequences for *IGF2* DMR used in the current study were taken from the original paper.[43] PCR reactions for *DNMT1, RXRA*, and *LEP* consisted of an initial hold at 95°C for 15 min followed by 45 cycles of 30s at 94°C, 30s at 54°C, and 30s at 72°C. PCR amplification ended with a final extension step at 72°C for 10 min. PCR reactions for *IGF2* DMR consisted of an initial hold at 5°C for 15 min followed by 5 cycles of 30s at 94°C, 30s at 68°C, and 30s at 72°C. This was followed by 50 cycles of 30s at 94°C, 30s at 64°C, and 30s at 72°C and ended with a final extension step at 72°C for 10 min. Primer information can be found in supplementary tables 1 and 2.

Pyrosequencing

In order to assess CpG methylation levels, 20 µL of biotinylated PCR product was immobilized to Streptavidin Sepharose High Performance beads (#17-5113-01, GE Healthcare) followed by annealing to 25 µL of 0.3 µM sequencing primer at 80°C for 2 min with a subsequent 10 min cooling down period. Pyrosequencing was performed using Pyro Gold reagents (#970802, Qiagen) on the PyroMark Q24 instrument (Qiagen) following the manufacturer's instructions. Pyrosequencing results were analyzed using the PyroMark analysis 2.0.7 software (Qiagen). Pyrosequencing provides information in about the methylation status of individual CpG sites and the average CpG methylation can be calculated.

Statistical analysis

First, we assessed the intake of dietary and supplemental maternal methyl-group donors before and during pregnancy using a multivariate regression model for longitudinal measurements with methyl-group donor intake as a response variable and time point as a factor (LSD post hoc test).

Next, we determined the effect of maternal dietary methyl-group donor intake on cord blood global DNA (hydroxy)methylation and gene specific methylation using linear regression models. Multivariable models were used to correct for possible covariates. Potential covariates were selected based on the association with DNA methylation and maternal nutrition: maternal age, maternal BMI, maternal smoking before and during each trimester of pregnancy (did not smoke /smoked), gestational weight gain. Analyses were performed separately per time point (pre-pregnancy, 11 - 13 weeks pregnancy, 18 - 22 weeks pregnancy, 30 - 34 weeks pregnancy). As high correlations were observed between methyl-group donor intakes at the different time points, it was less opportune to model the intake levels jointly in a multivariable model, given that highly correlated variables induce multicollinearity. Proportional odds models for ordinal data were used in case the response variable shows less than five levels. This was the case for the methylation percentage of DNMT1 CpG1,2,3, and 5.

Finally, we assessed whether there were differences in cord blood gene specific DNA methylation (*RXRA, IGF2 DMR, LEP, and DNMT1*) depending on the duration of maternal supplemental folic acid intake before and during pregnancy using one-Way ANOVA. Post hoc tests (Tukey test and Games Howell test (when the data did not meet the homogeneity of variances assumption)) were run when an overall significant difference in-group means was shown. For preconceptional supplemental folic acid intake, women were divided into 4 categories: no folic acid use before conception, start folic acid use 1 - 3 months prior to conception, 3 - 6 months prior to conception, or more than 6 months prior to conception. To test the effect of duration of supplemental folic acid use during pregnancy, women were divided into 3 categories: stop folic acid intake after the first trimester, stop after the second trimester, stop at the end of the third trimester.

All tests were two-sided, a 5 % significance level was assumed for all tests. Analyses were performed using SAS software (version 9.4 of the SAS System for Windows).

Conflict of interest: None

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Ethics: The current study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the UZ Leuven-Committee for Medical Ethics (reference number: ML7975). Written informed consent was obtained from all subjects.

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Figure 1. Cord blood *LEP* methylation by duration of maternal supplemental folic acid intake before conception. The bars represent the mean methylation values and standard errors of the mean of the 115 newborns. The results are based on the duration (4 categories) of maternal supplemental folic acid intake before conception. The overall p-values (One-way ANOVA) and significant p-values with mean differences from post-hoc tests are shown.

Figure 2. Cord blood *RXRA* methylation by duration of supplemental folic acid intake during pregnancy. The bars represent the mean methylation values and standard errors of the mean of 115 newborns. The results are based on the duration (3 categories) of maternal folic acid supplement intake during pregnancy. The overall p-values (One-way ANOVA) and significant p-values with mean differences from post-hoc tests are shown. MD; Mean Difference

Table 1. Characteristics of the mother-infant pairs included in the statistical analysis (n = 115)

Characteristics	Mean (SD)	Range
Mother		
Maternal age (y)	31 (3.6)	25 – 41
BMI (kg/m^2)	23.1 (3.4)	17.9 - 33
Gestational weight gain (kg)	14.8 (4.2)	1.9 - 28.9
Neonate		
Birth weight (gram)	3518 (405.4)	2720 – 4750
Gestational age (weeks)	39.6 (0.9)	37.1 – 41.4
Birth weight z-score	0.57 (0.93)	-1.38 – 2.91
	%	**n**
Maternal smoking (yes)		
Before pregnancy	4.3	5
First trimester	4.3	5
Second trimester	2.6	3
Third trimester	2.6	3
Gender newborn		
Boy	52.2	60
Girl	47.8	55

Table 2. Mean maternal intake of dietary methyl-group donors before and during pregnancy.

Methyl-group donor	**Before pregnancy Mean (SE) N = 24**	**First trimester (11 - 13w) Mean (SE) N = 96**	**Second trimester (18 - 22w) Mean (SE) N = 85**	**Third trimester (30 - 34w) Mean (SE) N = 83**
Methionine (mg)	1665.9 (468.2)	1662.8 (476.4)	1609.4 (450.8)	1625.9 (481.8)
Folate (µg)	271.4 (89.4)	275.4 (89.5)	263.2 (92.3)	273.4 (102.6)
Choline (mg)	285.9 (73.7)	278.9 (74.2)	271.4 (74.8)	273 (84.8)
Betaine (mg)	172.1 (63.7)	167.9 (59.5)	168.9 (61.6)	171.4 (62.5)

Multivariate regression model for longitudinal measurements

Table 3. Supplemental folic acid intake before and during pregnancy (n = 115).

	Mean (SE)	Range
Mean daily intake of folic acid (µg)		
Before pregnancy	371.5 (21.5)	0 – 1000
First trimester (11 - 13 weeks)	504.6 (14.1)*	171 – 1000
Second trimester (18 - 22 weeks)	386.9 (24.2)	0 – 1000
Third trimester (30 - 34 weeks)	354 (26.3)	0 – 1100
	%	**N**
Start folic acid use before pregnancy		
No	25.2	29
1 - 3 months	28.7	33
4 - 6 months	20.0	23
> 6 months	26.1	30
Folic acid use during pregnancy		
No	0	0
First trimester	18.3	21
Until second trimester	43.5	50
Whole pregnancy	38.3	44

*Significant higher folic acid intake in the first trimester of pregnancy (multivariate regression model for longitudinal measurements)

Table 4. Associations between maternal dietary methyl-group donor intake (before and during pregnancy) and offspring gene specific methylation (*LEP, DNMT1, RXRA*) measured in cord blood. Significant results are presented in bold.

Time point	**Before pregnancy N=24** β (95 % CI) p-value		**Second trimester N=89** β (95 % CI) p-value		**Third trimester N=83** β (95 % CI) p-value	
Gene / *Nutrient*	*LEP* *CpG4*	*DNMT1* *CpG4*	*LEP* *CpG2*	*DNMT1* *CpG4*	*RXRA* *CpG2*	*DNMT1* *CpG2*
Betaine	-0.13 (-3.45;3.19) 0.94	**0.675 (0.04;1.31) 0.039**	**-0.575 (-1.16;0.01) 0.05**	-0.25 (-0.58;0.09) 0.15	0.35 (-1.24;1.94) 0.66	0.97 (0.26;3.67) 0.96
Choline	1.48 (-1.48;4.45) 0.31	0.13 (-0.52;0.78) 0.68	-0.47 (-0.95;0.02) 0.058	**-0.301 (-0.57;-0.03) 0.031**	-0.935 (-2.08;0.21) 0.11	**0.291 (0.1;0.84) 0.022**
Folate	-0.33 (-2.75;2.09) 0.78	0.21 (-0.3;0.72) 0.4	**-0.507 (-0.89;-0.13) 0.009**	**-0.226 (-0.45;-0.01) 0.045**	**-1.001 (-1.96;-0.04) 0.041**	0.48 (0.22;1.06) 0.07
Methionine	**0.427 (0.01;0.85) 0.048**	0.04 (-0.06;0.14) 0.37	-0.06 (-0.14;0.02) 0.15	-0.04 (-0.08;0.009) 0.12	-0.15 (-0.35;0.06) 0.16	0.87 (0.74;1.04) 0.12

β-estimate is an absolute change in percentage of gene specific methylation; slope >(<) 0 means positive (negative) association; CI: confidence interval. Only the statistically significant associations are shown in this table.